

MODIFIED HIRUDIN PROTEINS AND T-CELL EPITOPES IN HIRUDIN**FIELD**

The invention concerns hirudin and in particular modified forms of hirudin with improved properties. The improved proteins contain amino acid substitutions at specific positions. The invention provides modified hirudin with improved biological activity concomitant with reduced immunogenic potential in the protein. The improved proteins are intended for therapeutic use in the treatment of diseases in humans.

10 BACKGROUND

Native hirudin constitutes a group of nearly identical polypeptides with a molecular weight around 7000 Daltons and consisting of 65 to 66 amino acids that are produced in the salivary glands of the leech *Hirudo medicinalis* [Markwardt F (1970) *Methods in Enzymology*; 19: 924-932]. Hirudin is the most potent natural inhibitor of the serine protease thrombin, with a
15 Ki of 10^{-11} to 10^{-14} M. It forms a 1:1 complex at the active site of thrombin resulting in inhibition of its proteolytic activity [Johnson PH, et al (1989) *Sem. Thromb. Hemost.*; 15: 320-315; Stone SR, & Hofsteenge J. (1986) *Biochemistry*; 4622-46242,3]. The X-ray crystal structure of hirudin and the hirudin/thrombin complex have been solved. The globular N-terminal domain of hirudin binds to the active-site cleft of thrombin, while the C-terminal tail
20 binds to the fibrinogen binding exosite of thrombin. Site directed mutagenesis studies have been carried out with both hirudin and the hirudin/thrombin complex [Grütter MG et al (1990) *EMBO Journal*; 9: 2361-2365; Rydel TJ, et al (1990) *Science*; 249:277-280; Rydel TJ, et al (1991) *Journal of Molecular Biology*; 221: 583-601].

25 Recombinant hirudins (r-hirudin) differ from naturally occurring hirudin by the lack of a sulphate group at tyrosine 63 and the first two N-terminal amino acids [Märki WE, et al (1997) *Semin. Thromb. Haemost.*; 17: 88-93]. Preclinical and clinical studies have shown that r-hirudin is an effective anticoagulant for prevention and treatment of venous thromboembolism in patients undergoing elective hip surgery and for patients with chronic stable coronary heart
30 disease, acute myocardial infarction, unstable angina pectoris as an adjunct to coronary angioplasty, and in combination with intracoronary thrombolysis [Lieber V, et al (2002) *Seminars in Thrombosis and Hemostasis*; 28: 483-489]. Recent studies have also suggested

that hirudin could be used in stroke therapy [Karabiyikoglu M, et al (2004) *Journal of Cerebral Blood Flow & Metabolism*. 24: 159-166].

The therapeutic potential of hirudin based thrombin inhibitors has been demonstrated by the development of Desirudin [Iprivask, Aventis Inc.]; [Val¹, Val²]-63-desulphohirudin and

5 Lepirudin [Refludan, Schering/Berlex]; [Leu¹, Thr²]-63-desulphohirudin.

Desirudin was first approved in the EU for thrombosis prophylaxis after orthopaedic hip and knee surgery. In April 2003 it was approved by the FDA and is now marketed as Iprivask.

Lepirudin is approved in both the European Union (1997) and the United States (1998) for the
10 treatment of patients suffering from heparin-induced thrombocytopenia (HIT) [Greinacher A, et al (1999). *Circulation*; 99: 73-80; Greinacher A, Janssens U, Berg G et al. (1999)

Circulation; 99: 587-593; Harenberg J, et al (1997) *Seminars in Thrombosis and Hemostasis*; 23: 189-196]. Heparin is currently used to prevent blood clots in more than 20 million patients per year in Europe and the US. In the US and EU alone, approximately 500,000 patients per

15 year are potential candidates for lepirudin therapy.

Despite its evident clinical utility, a significant problem associated with the clinical use of hirudin is its immunogenicity. Anti-hirudin antibodies can be detected in patients who have received lepirudin and desirudin [Song X, et al (1999) *Circulation*; 100: 1528-1532; Eichler

20 P, et al (2000) *Blood*; 96: 2373-2378; Greinacher A, et al (2003) *Blood*; 101: 2617-2619; Kischer K-G, et al (2003) *Thromb. Haemost*; 89: 973-982; Eichler P, et al (2004) *Blood*; 103: 613-616]. Moreover, lepirudin can cause fatal anaphylaxis, particularly in HIT patients who are treated within 3 months of a previous exposure. Between 1994 and 2002, 9 patients have been judged to have had severe anaphylaxis in close temporal contact with lepirudin. All

25 reactions occurred within minutes of intravenous lepirudin administration, with 4 fatal outcomes. In all 4 cases, a previous uneventful treatment course with lepirudin was identified (1 to 12 weeks earlier). High-titre IgG anti-lepirudin antibodies were recorded in an additional patient with anaphylaxis [Greinacher A, Lubenow N, Eichler P (2003). *Circulation*; 108: 2062-2065].

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Generation of a high affinity long lived antibody response, and in particular a response that can be seen to have undergone class switching to high titre IgG antibody, is dependent on CD4+ T-cell help. T cell receptor (TCR) binding of peptide epitopes presented in the context

of MHC Class II on the surface of antigen presenting cells (APCs), will lead to T-cell proliferation and the production of cytokines that can modulate immune responses. Patients who develop antibodies to hirudin□ have T-cells that are capable of recognising peptide fragments of hirudin bound to MHC class II molecules.

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The present invention is concerned with the identification of T-cell epitopes in hirudin and with hirudin molecules in which amino acid substitution and or combinations of substitution have been conducted. The substitutions confer a reduced immunogenic profile on the protein whilst retaining functional activity.

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SUMMARY OF THE INVENTION

The invention provides hirudin molecules containing amino acid substitutions. The amino acid substitutions confer improved properties to the protein. The improved properties concern the immunogenic properties of the protein.

The molecules of the invention have new properties. Such molecules may cause benefit for a patient in need of anticoagulation therapy or prophylaxis.

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The molecules of the invention are characterised by the protein sequences defined herein as M1-81.

The molecules of the invention are further characterised their relative activity in a thrombin inhibition assay of between around 0.2 and around 3.3.

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The most preferred molecules of the invention is characterised by the protein sequences M1 and M2 and are further characterised by a relative activity of around 1.2 – 1.4 in a thrombin inhibition assay.

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The most preferred molecules of the invention are characterised yet further still by comprising sequences demonstrated to show reduced immunogenicity in human cells. In particular

reduced immunogenicity as measured using a "T-cell assay" or a "time course assay" as defined herein.

5 The present invention provides for modified forms of hirudin proteins that are expected to display enhanced properties *in vivo*. The present invention discloses the major regions of the hirudin primary sequence that are immunogenic in man and provides modification to the sequences to eliminate or reduce the immunogenic effectiveness of these sites.

10 In one embodiment, synthetic peptides comprising the immunogenic regions can be provided in pharmaceutical composition for the purpose of promoting a tolerogenic response to the whole molecule.

In a further embodiment, the modified hirudin molecules of the present invention can be used in pharmaceutical compositions.

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In summary the invention is concerned with the following issues:

- A modified hirudin molecule having the biological activity of hirudin yet containing one or more amino acid substitutions;
- an accordingly specified molecule, wherein said amino acid substitutions are conducted
20 within the sequence (A) below comprising T-cell epitopes wherein;
A) = CILGSDGEKNQCVTGEGTPKPESHNDGDFE;
- an accordingly specified molecule, wherein said originally present T-cell epitope sequence (A) comprises MHC class II ligands or peptide sequences which show the ability to stimulate or bind T-cells via presentation on class II;
- 25 • a peptide sequence consisting of at least 9 consecutive amino acid residues as derived from sequence (A) above and its use for the manufacture of a hirudin like molecule having substantially no or less immunogenicity than any non-modified molecule and having the biological activity of hirudin when used *in vivo*;
- a pharmaceutical composition comprising 9 or more consecutive residues from the peptide
30 sequence (A) above;
- a modified hirudin molecule having the biological activity of hirudin and comprising one or more amino acid substitutions as specified above or below;

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- a modified hirudin molecule having the biological activity of hirudin and comprising at least the amino acid substitutions I29A and L30A;
- a modified hirudin molecule having the biological activity of hirudin and comprising at least the amino acid substitutions I29R and L30H;

- 5 • a modified hirudin molecule of structure (M):

V V Y T D C T E S G Q N X¹ C X² C E G S V X³ C G Q G N K C X⁴ X⁵ G S
D G E K N Q C X⁶ T G E G T P X⁷ X⁸ E S H N X⁹ G D X¹⁰ E E I P E E Y
L Q

wherein;

10 X¹ = T or L

X² = T or A or H or Q or T or L;

X³ = A or G or H or K or N or P or Q or R or V;

X⁴ = A or D or E or G or H or K or N or Q or R or S or T or I;

X⁵ = A or D or E or G or H or K or N or P or Q or R or S or T or L;

15 X⁶ = A or T or V;

X⁷ = T or K;

X⁸ = A or T or P;

X⁹ = E or N or R or D;

X¹⁰ = H or F

20 and whereby X¹ = L, X² = L, X³ = V, X⁴ = I, X⁵ = L, X⁶ = V, X⁷ = K, X⁸ = P, X⁹ = D and X¹⁰ = F are simultaneously excluded.

The mutant proteins of the present invention are readily made using recombinant DNA techniques well known in the art and the invention provides methods for the recombinant
25 production of such molecules.

In as far as this invention relates to modified hirudin, compositions containing such modified hirudin proteins or fragments of modified hirudin proteins and related compositions should be considered within the scope of the invention. In another aspect, the present invention relates
30 to nucleic acids encoding modified hirudin entities. In a further aspect the present invention relates to methods for therapeutic treatment of humans using the modified hirudin.

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DETAILED DESCRIPTION OF THE INVENTION

In nature, the mature hirudin protein is polypeptide of 65 to 66 amino acids. The amino acid sequence of a wild type (WT) hirudin (depicted as single-letter code) is as follows (M82) :

5 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C I L G S D G E
K N Q C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

The term "hirudin" is used herein to denote recombinant or synthetic equivalents of the above sequence which are in turn analogues of the native hirudin proteins constituting a group of
10 nearly identical polypeptides with a molecular weight around 7000 Daltons and which are produced in the salivary glands of the leech *Hirudo medicinalis*. In some instances the term is also used more broadly herein to include modified hirudin proteins or more especially a hirudin mutein.

15 The term "mutein" is used herein to denote a hirudin protein engineered to contain one or more amino acid substitutions differing from the above native sequence.

The term "peptide" as used herein, is a compound that includes two or more amino acids. The amino acids are linked together by a peptide bond.

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A peptide bond is the sole covalent linkage between amino acids in the linear backbone structure of all peptides, polypeptides or proteins. The peptide bond is a covalent bond, planar in structure and chemically constitutes a substituted amide. An "amide" is any of a group of organic compounds containing the grouping -CONH-.

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There are 20 different naturally occurring amino acids involved in the biological production of peptides, and any number of them may be linked in any order to form a peptide chain or ring. The naturally occurring amino acids employed in the biological production of peptides all have the L-configuration. Synthetic peptides can be prepared employing conventional
30 synthetic methods, utilizing L-amino acids, D-amino acids, or various combinations of amino acids of the two different configurations. Some peptides contain only a few amino acid units. Short peptides, e.g., having less than ten amino acid units, are sometimes referred to as "oligopeptides". Other peptides contain a large number of amino acid residues, e.g. up to 100

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or more, and are referred to as "polypeptides". By convention, a "polypeptide" may be considered as any peptide chain containing three or more amino acids, whereas a "oligopeptide" is usually considered as a particular type of "short" polypeptide. Thus, as used herein, it is understood that any reference to a "polypeptide" also includes an oligopeptide.

5 Further, any reference to a "peptide" includes polypeptides, oligopeptides, and proteins. Each different arrangement of amino acids forms different polypeptides or proteins. The number of polypeptides—and hence the number of different proteins—that can be formed is practically unlimited.

10 Since the peptide bond is the sole linkage between amino acids, all peptides, polypeptides or proteins have defined termini conventionally referred to as the "N-terminus" or "N-terminal" residue and the "C-terminus" or "C-terminal residue". The N-terminal residue bears a free amino group, whereas the C-terminal residue bears a free carboxyl group.

15 The term "T-cell epitope" means according to the understanding of this invention an amino acid sequence which is able to bind MHC class II, able to stimulate T-cells and / or also to bind (without necessarily measurably activating) T-cells in complex with MHC class II. In principle such an amino acid sequence can be not less than 9 residues in length but typically will be 10 or 11 or 12 or 13 or 14 or 15 or more residues in length.

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Reference to "substantially non-immunogenic" or "reduced immunogenic potential" includes reduced immunogenicity compared to a parent protein or to a fusion protein containing the wild-type (WT) or native amino acid sequences of the test moiety.

25 The term "immunogenicity" includes an ability to provoke, induce or otherwise facilitate a humoral and or T-cell mediated response in a host animal and in particular where the "host animal" is a human.

The terms "T-cell assay" and "immunogenicity assay" concern *ex vivo* measures of immune
30 reactivity. As such these involve a test immunogen e.g. a protein or peptide being brought into contact with live human immune cells and their reactivity measured. A typical parameter of induced reactivity is proliferation. The presence of suitable control determinations are critical and implicit in the assay.

“Time course assay” refers to a biological assay such as a proliferation assay in which determinations of activity are made sequentially over a period of time. In the present context, a “time course T-cell assay”, refers to the determination of T-cell proliferation in response to a test immunogen (peptide) at multiple times following exposure to the test immunogen. The terms “time course T-cell assay” and “time course immunogenicity assay” may be used interchangeably herein.

One conventional way in which T-cell assays are expressed is by use of a “stimulation index” or “SI”. The stimulation index (SI) is conventionally derived by division of the proliferation score (e.g. counts per minute of radioactivity if using for example ^3H -thymidine incorporation) measured to a test immunogen such as a peptide by the score measured in cells not contacted with a test immunogen. Test immunogens (peptides) which evoke no response give $\text{SI} = 1.0$ although in practice SI values in the range 0.8 - 1.2 are unremarkable. The inventors have established that in the operation of such immunogenicity assays, a stimulation index equal to or greater than 2.0 is a useful measure of significant induced proliferation.

PBMC means peripheral blood mononuclear cells in particular as obtained from a sample of blood from a donor. PBMC are readily isolated from whole blood samples using a density gradient centrifugation technique well understood in the art and comprise predominantly lymphocytes (B and T cells) and monocytes. Other cell types are also represented.

“Relative activity” means according to the present context activity measured for a test protein in any single assay expressed relative to the activity measured for a positive control protein in an identical assay and usually conducted in parallel. Thus if the test protein and the control protein have the same measured activity the relative activity is said to be 1.

A “thrombin inhibition assay” according to the present context means an *in vitro* assay able to provide a reading of the functional capability of the test protein. In the present instance this means the ability of a given hirudin mutein to evoke a specific measurable chromogenic response. Particularly suitable assays are exemplified herein using thrombin and the chromogenic thrombin substrate H-D-Phe-Pep-Arg-pNA. Other assay formats can be contemplated to also provide quantitative estimations of specific activity of the test molecules

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and permit ED₅₀ determinations. Examples include the Activated Partial Thromboplastin Time coagulation (aPTT) assay and the Ecarin clotting time (ECT) assay, each well known in the art.

5 In another aspect, the present invention relates to nucleic acids encoding modified hirudin entities. Such nucleic acids are preferably comprised within an expression vector. The control sequences ~~that are suitable for prokaryotes, for example, include a promoter, optionally an~~ operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilise promoters, enhancers and polyadenylation signals. Such nucleic acids in general comprise a selection means typically an additional gene encoding a protein able to provide for the survival
10 of the host cell. An example of such a selection gene is the beta-lactamase gene suitable for some *E. coli* host cells and this and others are well known in the art ["Molecular Cloning: A Laboratory Manual", second edition (Sambrook et al., 1989); "Gene Transfer Vectors for Mammalian Cells" (J. M. Miller & M. P. Calos, eds., 1987); "Current Protocols in Molecular
15 Biology" (F. M. Ausubel et al., eds., 1987)].

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a pre-sequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the
20 secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in the same reading frame. However, enhancers do not have to be
25 contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

In some embodiments the expression vector comprises a nucleic acid sequence encoding a
30 hirudin variant operably linked to an expression control sequence. In various embodiments the expression vector comprises a nucleic acid sequence encoding a protein selected from the group comprising inclusively M1 to M81. Such an expression vector will comprise at least the hirudin encoding domain of one of the said proteins operably linked with suitable expression

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control and selection sequences. Such an expression vector would include degenerate versions of the nucleic acid wherein degeneracy in relation to polynucleotides refers to the fact well recognised that in the genetic code many amino acids are specified by more than one codon. The degeneracy of the code accounts for 20 different amino acids encoded by 64 possible triplet sequences of the four different bases comprising DNA.

Another aspect of the present invention is a cultured cell comprising at least one of the above-mentioned vectors.

10 A further aspect of the present invention is a method for preparing the modified hirudin comprising culturing the above mentioned cell under conditions permitting expression of the hirudin from the expression vector and purifying the hirudin from the cell.

Whilst the hirudin molecules of the invention and are readily made using the above described recombinant DNA based methods, it is recognised that as short polypeptides, the preferred molecules of the invention can be made by wholly synthetic means using solid phase chemical synthesis methods or equivalent techniques well known in the art [for review see Bruckdorfer, T. et al (2004) *Curr. Pharm. Biotechnol.* 5:29-43 and references therein].

20 In a yet further aspect, the present invention relates to methods for therapeutic treatment of humans using the hirudin compositions. For administration to an individual, any of the modified compositions would be produced to be preferably at least 80% pure and free of pyrogens and other contaminants. It is further understood that the therapeutic compositions of the hirudin proteins may be used in conjunction with a pharmaceutically acceptable excipient.

25 The pharmaceutical compositions according to the present invention are prepared conventionally, comprising substances that are customarily used in pharmaceuticals, e.g. Remington's Pharmaceutical Sciences, (Alfonso R. Gennaro ed. 18th edition 1990), including excipients, carriers adjuvants and buffers. The compositions can be administered, e.g. parenterally, enterally, intramuscularly, subcutaneously, intravenously or other routes useful to

30 achieve an effect. Conventional excipients include pharmaceutically acceptable organic or inorganic carrier substances suitable for parenteral, enteral and other routes of administration that do not deleteriously react with the agents. For parenteral application, particularly suitable are injectable sterile solutions, preferably oil or aqueous solutions, as well as suspensions,

emulsions or implants, including suppositories. Ampules are convenient unit dosages. The pharmaceutical preparations can be sterilised and, if desired, mixed with stabilisers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers or other substances that do not react deleteriously with the active compounds.

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The major embodiments of the present invention are encompassed by the protein sequences M 1 – 81. A number of muteins show improved activity relative to the WT molecule (M82). All active muteins are embodiments of the invention.

- 10 The hirudin muteins of the present were constructed to be less immunogenic than the parental molecule. The design of individual muteins was directed from immunological considerations as well as functional activity data. A single major region of immunological importance within the molecule was defined using screening assays involving use of PBMC preparations from healthy donor subjects. This approach has proven to be a particularly effective method for the
- 15 identification such biologically relevant immunogenic peptides and is disclosed herein as an embodiment of the invention. In the present study, the method has involved the testing of overlapping hirudin-derived peptide sequences in a scheme so as to scan and test the entire hirudin sequence. Such a scan required synthesis and use of 19 peptides each of 15 residues in length. The synthetic peptides were tested for ability to evoke a proliferative response in
- 20 human T-cells cultured *in vitro*. Where this type of approach is conducted using naïve human T-cells taken from healthy donors, the inventors have established that a stimulation index equal to or greater than 2.0 is a useful measure of induced proliferation.

- One significant epitope region (termed R1) was identified in these studies. R1 encompasses
- 25 residues 28 – 57 and comprises the sequence CILGSDGEKNQCVTGEGTPKPESHNDGDFE.

- The R1 peptide sequence represents the critical information required for the construction of modified hirudin molecules in which the epitope is compromised. Equally, the R1 sequence represent the critical information required for the production of tolerogenic peptides. Epitope
- 30 region R1 is an embodiment of the invention.

Under the scheme of the present, the epitopes are compromised by mutation to result in sequences no longer able to function as T-cell epitopes. It is possible to use recombinant DNA

methods to achieve directed mutagenesis of the target sequences and many such techniques are available and well known in the art. Broadly, the hirudin muteins herein were constructed containing mutations within the immunogenic region R1. Individual residues were targeted based upon the known binding properties of HLA-DR molecules in that they have an almost
 5 exclusive preference for a hydrophobic amino acid in pocket 1 and that this is the most important determinant of peptide binding [Jardetzky, T.S. et al (1990), *EMBO J.* 9: 1797-1803; Hill, C.M. et al (1994) *J. Immunol.* 152: 2890-2898]. Exhaustive mutational analysis identified those residues both inside and outside R1 that could be altered without adversely affecting the activity of the fusion protein. A number of muteins show improved activity
 10 relative to the WT molecule. All active muteins are embodiments of the invention.

The general method of the present invention leading to the modified TPO comprises the following steps:

- (a) determining the amino acid sequence of the polypeptide or part thereof;
- 15 (b) identifying one or more potential T-cell epitopes within the amino acid sequence of the protein by any method including determination of the binding of the peptides to MHC molecules using *in vitro* or *in silico* techniques or biological assays;
- (c) designing new sequence variants with one or more amino acids within the identified potential T-cell epitopes modified in such a way to substantially reduce or eliminate the
 20 activity of the T-cell epitope as determined by the binding of the peptides to MHC molecules using *in vitro* or *in silico* techniques or biological assays. Such sequence variants are created in such a way to avoid creation of new potential T-cell epitopes by the sequence variations unless such new potential T-cell epitopes are, in turn, modified in such a way to substantially reduce or eliminate the activity of the T-cell epitope; and
- 25 (d) constructing such sequence variants by recombinant DNA techniques and testing said variants in order to identify one or more variants with desirable properties according to well known recombinant techniques.

Taken together, the inventors have been able to define modified hirudin proteins which can be
 30 depicted by the following structure (M):

V V Y T D C T E S G Q N X¹ C X² C E G S V X³ C G Q G N K C X⁴ X⁵ G S D
 G E K N Q C X⁶ T G E G T P X⁷ X⁸ E S H N X⁹ G D X¹⁰ E E I P E E Y L Q
 wherein;

$X^1 = T \text{ or } L$

$X^2 = T \text{ or } A \text{ or } H \text{ or } Q \text{ or } T \text{ or } L;$

$X^3 = A \text{ or } G \text{ or } H \text{ or } K \text{ or } N \text{ or } P \text{ or } Q \text{ or } R \text{ or } V;$

$X^4 = A \text{ or } D \text{ or } E \text{ or } G \text{ or } H \text{ or } K \text{ or } N \text{ or } Q \text{ or } R \text{ or } S \text{ or } T \text{ or } I;$

5 $X^5 = A \text{ or } D \text{ or } E \text{ or } G \text{ or } H \text{ or } K \text{ or } N \text{ or } P \text{ or } Q \text{ or } R \text{ or } S \text{ or } T \text{ or } L;$

$X^6 = A \text{ or } T \text{ or } V;$

$X^7 = T \text{ or } K;$

$X^8 = A \text{ or } T \text{ or } P;$

$X^9 = E \text{ or } N \text{ or } R \text{ or } D;$

10 $X^{10} = H \text{ or } F$

and whereby $X^1 = L$, $X^2 = L$, $X^3 = V$, $X^4 = I$, $X^5 = L$, $X^6 = V$, $X^7 = K$, $X^8 = P$, $X^9 = D$ and $X^{10} = F$ are simultaneously excluded.

15 The following, figures, sequence listing and examples are provided to aid the understanding of the present invention. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

DESCRIPTION OF THE SEQUENCES

20 To aid the understanding of the invention, Table 1 below sets out a description of the hirudin muteins. The derivation and properties of these proteins are also more fully disclosed in the examples.

Table 1

25 **Description of the sequences**

M No	Substitution(s)	M No	Substitution(s)
M 1	I29A L30A	M 42	V21A
M 2	I29R L30H	M 43	V21G
M 3	I29E L30K V40A	M 44	V21H
M 4	I29E L30K V40T	M 45	V21K
M 5	I29E L30K D53E	M 46	V21N
M 6	I29E L30K D53N	M 47	V21P
M 7	I29R L30K V40A	M 48	V21Q
M 8	I29R L30K V40T	M 49	V21R
M 9	I29R L30K D53E	M 50	I29A

M 10	I29R L30K D53N	M 51	I29D
M 11	I29R L30R V40T	M 52	I29E
M 12	I29A L30K	M 53	I29G
M 13	I29A L30Q	M 54	I29H
M 14	I29A L30R	M 55	I29K
M 15	I29A L30T	M 56	I29N
M 16	I29D L30A	M 57	I29Q
M 17	I29D L30Q	M 58	I29R
M 18	I29D L30R	M 59	I29S
M 19	I29E L30K	M 60	I29T
M 20	I29E L30Q	M 61	L30A
M 21	I29E L30R	M 62	L30D
M 22	I29E L30T	M 63	L30E
M 23	I29R L30K	M 64	L30G
M 24	I29R L30Q	M 65	L30H
M 25	I29R L30R	M 66	L30K
M 26	I29R L30T	M 67	L30N
M 27	I29S L30A	M 68	L30P
M 28	I29S L30K	M 69	L30Q
M 29	I29S L30Q	M 70	L30R
M 30	I29S L30R	M 71	L30S
M 31	I29S L30T	M 72	L30T
M 32	I29T L30A	M 73	V40A
M 33	I29T L30K	M 74	V40T
M 34	I29T L30Q	M 75	K47T
M 35	I29T L30R	M 76	P48A
M 36	I29T L30T	M 77	P48T
M 37	L13T	M 78	D53E
M 38	L15A	M 79	D53N
M 39	L15H	M 80	D53R
M 40	L15Q	M 81	F56H
M 41	L15T	M 82	WT

Table A

Sequences of modified (M1 – M81) and wild-type hirudin (M82), and T-cell epitope containing region A within wild-type hirudin:

5

M1

V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C A A G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

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M2

V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C R H G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

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M3

V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C E K G S D G E K N Q
C A T G E G T P K P E S H N D G D F E E I P E E Y L Q

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M4
V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C E K G S D G E K N Q
C T T G E G T P K P E S H N D G D F E E I P E E Y L Q

5 M5
V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C E K G S D G E K N Q
C V T G E G T P K P E S H N E G D F E E I P E E Y L Q

10 M6
V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C E K G S D G E K N Q
C V T G E G T P K P E S H N N G D F E E I P E E Y L Q

15 M7
V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C R K G S D G E K N Q
C A T G E G T P K P E S H N D G D F E E I P E E Y L Q

20 M8
V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C R K G S D G E K N Q
C T T G E G T P K P E S H N D G D F E E I P E E Y L Q

M9
V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C R K G S D G E K N Q
C V T G E G T P K P E S H N E G D F E E I P E E Y L Q

25 M10
V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C R K G S D G E K N Q
C V T G E G T P K P E S H N N G D F E E I P E E Y L Q

30 M11
V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C R R G S D G E K N Q
C T T G E G T P K P E S H N D G D F E E I P E E Y L Q

35 M12
V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C A K G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

40 M13
V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C A Q G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M14
V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C A R G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

45 M15
V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C A T G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

50 M16
V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C D A G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

55 M17
V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C D Q G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M18
V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C D R G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

60

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M 19

V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C E K G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

5 M 20

V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C E Q G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 21

10 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C E R G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 22

15 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C E T G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 23

20 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C R K G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 24

V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C R Q G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

25 M 25

V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C R R G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 26

30 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C R T G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 27

35 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C S A G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 28

40 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C S K G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 29

V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C S Q G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

45 M 30

V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C S R G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 31

50 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C S T G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 32

55 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C T A G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 33

60 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C T K G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

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M 34

V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C T Q G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

5 M 35

V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C T R G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 36

10 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C T T G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 37

15 V V Y T D C T E S G Q N T C L C E G S V V C G Q G N K C I L G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 38

20 V V Y T D C T E S G Q N L C A C E G S V V C G Q G N K C I L G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 39

V V Y T D C T E S G Q N L C H C E G S V V C G Q G N K C I L G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

25 M 40

V V Y T D C T E S G Q N L C Q C E G S V V C G Q G N K C I L G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 41

30 V V Y T D C T E S G Q N L C T C E G S V V C G Q G N K C I L G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 42

35 V V Y T D C T E S G Q N L C L C E G S V A C G Q G N K C I L G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 43

40 V V Y T D C T E S G Q N L C L C E G S V G C G Q G N K C I L G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 44

V V Y T D C T E S G Q N L C L C E G S V H C G Q G N K C I L G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

45 M 45

V V Y T D C T E S G Q N L C L C E G S V K C G Q G N K C I L G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 46

50 V V Y T D C T E S G Q N L C L C E G S V N C G Q G N K C I L G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 47

55 V V Y T D C T E S G Q N L C L C E G S V P C G Q G N K C I L G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 48

60 V V Y T D C T E S G Q N L C L C E G S V Q C G Q G N K C I L G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

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M 49

V V Y T D C T E S G Q N L C L C E G S V R C G Q G N K C I L G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

5 M 50

V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C A L G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 51

10 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C D L G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 52

15 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C E L G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 53

20 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C G L G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 54

V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C H L G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

25 M 55

V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C K L G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 56

30 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C N L G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 57

35 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C Q L G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 58

40 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C R L G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 59

V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C S L G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

45 M 60

V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C T L G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 61

50 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C I A G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 62

55 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C I D G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 63

60 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C I E G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

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M 64

V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C I G G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

5 M 65

V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C I H G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 66

10 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C I K G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 67

15 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C I N G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 68

20 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C I P G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 69

V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C I Q G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

25 M 70

V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C I R G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 71

30 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C I S G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 72

35 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C I T G S D G E K N Q
C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 73

40 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C I L G S D G E K N Q
C A T G E G T P K P E S H N D G D F E E I P E E Y L Q

M 74

V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C I L G S D G E K N Q
C T T G E G T P K P E S H N D G D F E E I P E E Y L Q

45 M 75

V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C I L G S D G E K N Q
C V T G E G T P T P E S H N D G D F E E I P E E Y L Q

M 76

50 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C I L G S D G E K N Q
C V T G E G T P K A E S H N D G D F E E I P E E Y L Q

M 77

55 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C I L G S D G E K N Q
C V T G E G T P K T E S H N D G D F E E I P E E Y L Q

M 78

60 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C I L G S D G E K N Q
C V T G E G T P K P E S H N E G D F E E I P E E Y L Q

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M 79

V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C I L G S D G E K N Q
 C V T G E G T P K P E S H N N G D F E E I P E E Y L Q

5 M 80

V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C I L G S D G E K N Q
 C V T G E G T P K P E S H N R G D F E E I P E E Y L Q

M 81

10 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C I L G S D G E K N Q
 C V T G E G T P K P E S H N D G D H E E I P E E Y L Q

M 82

15 V V Y T D C T E S G Q N L C L C E G S V V C G Q G N K C I L G S D G E K N Q
 C V T G E G T P K P E S H N D G D F E E I P E E Y L Q

(A):

C I L G S D G E K N Q C V T G E G T P K P E S H N D G D F E

20

DESCRIPTION OF THE FIGURES

Figure 1:

The mature sequence of [Val¹ Val²]-hirudin with regions of immunogenicity highlighted in bold. The most preferred modifications are to residues 29 and 30 (underlined). The preferred alternative substitutions are indicated above the sequence.

Figure 2:

Immunogenicity of [Val¹ Val²]-hirudin variant peptides. Two cohorts of 20 healthy donors were used to test the immunogenicity of wild type region 1 and modified region 1 peptides. Proliferation of PBMC was assessed by tritiated thymidine incorporation on days 6, 7, 8 and 9 post-stimulation and stimulation indexes were calculated and plotted.

Figure 3:

Frequency of observed responses with an SI>2 at any time point from cohorts of 20 healthy donors to either wild type [Val¹ Val²]-hirudin region 1 and modified R1 peptides.

Figure 4:

Purification of [Val¹ Val²]-hirudin from transfected HEK.293 cell supernatants. Elution of pooled fractions from pH 7.0 elution on Mono Q column at pH 5.5. Main peak containing hirudin is marked with an arrow.

Figure 5:

Silver stained SDS-PAGE gel of fractions from elution of Mono Q column at pH 5.5. Lane 1= diluted supernatant loaded onto Mono Q column at pH 7.0; Lane 2 = Concentrated fractions

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from elution of Mono Q column at pH 7.0; Lane 3 = Protein Standards; Lane 4 = Fraction 1 pH 5.5 elution; Lane 5 = Fraction 10 pH 5.5 elution; Lane 6 = Fraction 14 pH 5.5 elution; Lane 7 = Fraction 15 pH 5.5 elution; Lane 8 = Fraction 17 pH 5.5 elution; Lane 9 = Fraction 19 pH 5.5 elution; Lane 10 = Fraction 22 pH 5.5 elution; Lane 11 = Protein Standards. Bands corresponding to hirudin in lanes 6 and 7 are marked with an arrow.

Figure 6:

Thrombin inhibitory activity of the hirudin variants: I29A L30A and I29R L30H (M 1 and M 2 respectively). Cell-culture supernatants from I29A L30A(♦), I29R L30H (■) and WT hirudin (▲) from transfected HEK.293 cells, were compared in the chromogenic thrombin cleavage assay. The amount of hirudin variants in tissue culture supernatants were quantified by ELISA, and the thrombin inhibition activity was titrated in 2 fold serial dilutions.

EXPERIMENTAL EXAMPLES**EXAMPLE 1****Construction of hirudin muteins**

The cDNA for Hirudin was cloned from a cDNA library obtained by RT-PCR from mRNA extracted from the head of the medicinal leech, *Hirudo medicinalis* (Biopharm Ltd., Hendy, UK) using a Qiagen total RNA extraction kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. This sequence was mutated to that of [Val¹ Val²]-hirudin, by site directed mutagenesis PCR [Higuchi et al (1988) *Nucleic Acids Res.* 16: 7351].

For protein expression, the native 20 amino acid secretion signal,

(MFSLKLFVVFLAVCICVSQA, of a hirudin variant from the Asian buffalo leech (*Hirudinaria manillensis*) was added by PCR and this construct was then sub-cloned into the expression vector pREP4 (Invitrogen, Paisley, UK). Later a modified secretion signal

(MVSLKLFVVFLAVCICVSQA) was used in order to create a more efficient Kozak consensus sequence for protein expression in mammalian cell lines [Kozak M. (1987) *Nucleic Acids Research*; 15: 8125-8148; Kozak M. (1991) *Journal of Cell Biology*; 115: 887-903; Kozak M. (1990) *Proc. Natl. Acad. Sci. (USA)*; 87: 8301-8305], where the mutation of F² (TTC) to a V (GTC) placed a guanosine residue at position +4 in the consensus sequence.

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A total of 107 different hirudin muteins were constructed using PCR mutagenesis and the WT gene as a template. DNA sequencing was conducted on all constructs. This was diligently performed to confirm introduction of desired substitutions and establish that no extraneous (undesired) substitutions had been introduced for example by PCR error.

5

EXAMPLE 2

Expression and purification of hirudin proteins

Expression plasmids containing hirudin mutein genes were transfected into the human embryonic kidney cell line HEK.293. Transfection was conducted using lipofectamine
10 transfection reagent and cells were cultured in DMEM media free of phenol red (Invitrogen, Paisley, UK). Hirudin was purified from the culture media. Briefly, tissue culture supernatant was diluted 1:1 with Mono Q buffer A (20mM Bis-Tris-Propane pH 7.0). This was then filtered through a 0.22 µm membrane and applied to a Mono Q (HR5/5) column and protein was eluted using a 0-100% NaCl gradient in buffer A. Fractions were then tested for the
15 presence of hirudin by ELISA.

Hirudin enriched fractions were pooled and concentrated using a Centricon centrifugal concentrator fitted with a YM3 membrane (3kDa MW cut off). The pooled and concentrated fractions were buffer exchanged (G25 column) into buffer B (20mM Piperazine pH 5.5) and applied to a Mono Q column equilibrated with buffer B. Protein was eluted with a gradient
20 from 0-100% 0.5M NaCl in buffer B and was monitored at 214nm. Peak fractions were analysed on a 4-12% Bis-Tris SDS-PAGE gel (Invitrogen, Paisley, UK) using MES buffer. The gel was then silver stained to check the purity of the fractions.

EXAMPLE 3

25 Detection and quantitation of hirudin variants

Expressed hirudin was detected and quantitated using an ELISA with a matched pair of anti-hirudin antibodies [Koch C, et al (1993) *Analytical Biochemistry*; 214: 301-312]. Hirudin purified from *Hirudo medicinalis* saliva (Roche, Lewes, UK) was used as a standard in order to quantify expression. Briefly, a 96 well plate was coated with 100 µl of mouse anti-hirudin
30 N-terminal epitope (Antibody shop, Copenhagen, Denmark) at 10 µg/ml in PBS, overnight at 4°C. The plate was washed once with PBS/0.05% Tween, 200µl per well, then blocked with 200µl per well of PBS/2% BSA for one hour at room temperature. The plate was then washed 5x with PBS/0.05% Tween, 200µl per well, then blotted onto pad of paper tissues to remove

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excess liquid. A hirudin standard curve starting with 1280ng/ml was set up using doubling dilutions vertically down the plate. Hirudin variant samples were added in triplicate to a final volume of 100 µl in PBS at dilutions of 1:50 and 1:100. The plate was incubated for 1 hr at room temperature then washed 5x as described above. 100 µl of biotinylated mouse anti-
5 hirudin C-terminal epitope (Antibody Shop, Copenhagen, Denmark) was added at a 1:1000 dilution in PBS/2% BSA to each well and the plate incubated for 1 hr at room temperature. The plate was washed 5x as described above. 100 µl of streptavidin peroxidase (Sigma, Poole, UK) 1:1000 dilution in PBS/2% BSA was added and the plate was incubated for 1 hr at room temperature. The plate was washed as described above and 100 µl of o-phenylenediamine
10 dihydrochloride substrate (Sigma, Poole, UK) was added to each well and the plate incubated at room temperature for 5 minutes. The reaction was quenched by adding 50 µl of 1M H₂SO₄ to each well and the absorbance measured at 492nm.

EXAMPLE 4

15 Functional activity of hirudin muteins

The functional activity of the modified hirudin proteins was assessed using a chromogenic thrombin inhibition assay. In this assay, a fixed amount of thrombin is incubated with a range of hirudin concentrations at 37°C. The chromogenic substrate H-D-Phe-Pep-Arg-pNA is then added and any residual free thrombin present will cleave the substrate to form H-D-Phe-Pep-
20 Arg-OH and pNA that can then be detected spectrophotometrically at 405nm

Briefly, 2ml of dH₂O was added to the thrombin vial and the H-D-Phe-Pep-Arg-pNA substrate vial from a Diagnostica Stago Stachrom HCII assay kit (Axis Shield Diagnostics, Dundee, UK) and they were both warmed in an incubator to 37°C. Hirudin assay buffer (175mM
25 NaCl, 7.5 mM Na₂ EDTA, 50mM Tris-HCl pH 8.4) was warmed to 37°C then used to dilute the test hirudin protein variants, hirudin wild type and hirudin control (Roche, Lewes, UK) proteins.

Aliquots of 40µl of warm hirudin assay buffer were pipetted into wells A-H 2-12 of a clear 96 well flat bottom plate, then 40µl aliquots of hirudin samples were placed into the wells in
30 columns 1 and 2. Using a multichannel mix by pipetting up and down the contents of wells in column 2 and prepare a serial doubling dilution of the samples were made horizontally across the plate by removing 40µl from column 2 and adding to column 3 etc.

The plate was warmed to 37°C for 5 minutes followed by addition of 40µl thrombin and incubated for 120 seconds at 37°C with shaking. 40µl H-D-Phe-Pep-Arg-pNA substrate was added, the plate and incubated for 90 seconds at 37°C with shaking. 40µl acetic acid was added to quench reaction and the plate was read spectrophotometrically at 405nm.

Absorbance at 405nm vs hirudin concentration was plotted using Sigma plot (Sigma Poole, UK) and IC₅₀ values calculated by fitting a sigmoidal equation.

A total of 81 different hirudin variants demonstrated positive activity in the chromogenic thrombin inhibition assay. Others either failed to express or showed no acceptable degree of activity. Positive activity was taken to be a relative activity value of less than 10. Relative activity was determined by dividing the ED₅₀ value derived for the protein of interest by the ED₅₀ value derived for the control (WT) protein.

Of these active proteins, 45 were muteins comprising a single amino acid substitution; 27 comprised two amino acid substitutions and 9 comprised three amino acid substitutions. The sequence of each of these active hirudin muteins is provided in M 1 - 81. The relative activities of each functioning mutein are provided in Table 2.

A number of muteins show improved activity relative to the WT molecule. All active muteins are embodiments of the invention.

Table 2

Activity of hirudin variants

Substitution	Relative Activity*
L13T	0.60
L15A	0.90
L15H	0.65
L15Q	0.34
L15T	0.40
V21A	0.93
V21G	1.0
V21H	0.74
V21K	0.90
V21N	0.83
V21P	1.0
V21Q	0.83
V21R	0.77
I29A	1.0
I29D	1.0
I29E	1.0

Substitution	Relative Activity*
D53E	1.0
D53N	1.0
D53R	1.0
F56H	0.80
I29A L30A	1.40
I29A L30K	1.45
I29A L30Q	1.60
I29A L30R	2.70
I29A L30T	1.21
I29D L30A	1.60
I29D L30Q	2.0
I29D L30R	2.5
I29E L30K	1.10
I29E L30Q	1.52
I29E L30R	1.21
I29E L30T	1.80

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I29G	1.8
I29H	1.0
I29K	1.0
I29N	1.0
I29Q	1.0
I29R	1.0
I29S	1.0
I29T	1.0
L30A	1.0
L30D	2.0
L30E	3.33
L30G	2.20
L30H	1.0
L30K	1.0
L30N	1.54
L30P	1.83
L30Q	1.0
L30R	1.0
L30S	1.83
L30T	1.0
V40A	1.0
V40T	1.0
K47T	2.2
P48A	1.0
P48T	1.0

I29R L30H	1.2
I29R L30K	0.93
I29R L30Q	1.0
I29R L30R	1.0
I29R L30T	1.0
I29S L30A	1.34
I29S L30K	1.50
I29S L30Q	2.0
I29S L30R	2.5
I29S L30T	1.10
I29T L30A	1.67
I29T L30K	1.60
I29T L30Q	2.4
I29T L30R	2.30
I29T L30T	1.80
I29R L30K	0.20
I29R L30K	0.36
I29R L30R	0.70
I29E L30K	1.0
I29E L30K	1.0
I29E L30K	0.55
I29E L30K	1.0
I29R L30K	0.70
I29R L30K	0.80

*Ratio of variant IC₅₀ / WT IC₅₀

EXAMPLE 5

5 Identification of T- cell epitopes in hiridin □

All blood samples used in this study were obtained with approval of the Addenbrooke's Hospital Local Research Ethics Committee. T-cell epitope mapping was performed using human PBMCs isolated from blood obtained from the National Blood Transfusion Service (Addenbrooke's Hospital, Cambridge, UK). PBMCs from 25 healthy donors were isolated by Ficoll density centrifugation and stored under liquid nitrogen. Each donor was tissue-typed using an Allset™ PCR based tissue-typing kit (Dynal) and T cell assays were performed by selecting donors according to individual MHC haplotypes. 15mer peptides staggered by three amino acids and spanning the human hirudin sequence were purchased from Pepscan Systems BV (NL). Using this scheme, total of 19 peptides were required to scan the hirudin protein.

15 The sequence and peptide number of these peptides are provided in Table 3.

Table 3**Peptides used to map immunogenic epitopes within hirudin**

Peptide No	Peptide sequence	Peptide No	Peptide sequence
1	VVYTDCTESGQNLCL	11	CILGSDGEKNQCVTG
2	LTYTDCTESGQNLCL	12	GSDGEKNQCVTGEGT
3	TDCTESGQNLCLCEG	13	GEKNQCVTGEGTPKP
4	TESGQNLCLCEGSNV	14	NQCVTGEGTPKPESH
5	GQNLCLCEGSNVCGQ	15	VTGEGTPKPESHNDG
6	LCLCEGSNVCGQGK	16	EGTPKPESHNDGDGE
7	CEGSNVCGQGKNCIL	17	PKPESHNDGDGEFEEIP
8	SNVCGQGKNCILGSD	18	ESHNDGDGEFEEIPPEY
9	CGQGKNCILGSDGEK	19	NDGDGEFEEIPPEYYLQ
10	GNKNCILGSDGEKNQC		

For each donor sample, PBMCs were thawed and resuspended in AIM-V (Invitrogen)

5 containing 100 units/ml penicillin, 100ug/ml streptomycin and 1mM glutamine. Triplicate cultures of 2×10^5 PBMC/well of flat-bottomed 96 well plate were incubated with peptides at a final concentration of 1μM and 10μM. Cells were incubated for 7 days before pulsing with 1μCi/well tritiated thymidine for 18 hours. Cultures were harvested onto glass fibre filter mats using a Tomtec Mach III plate harvester and cpm values determined by scintillation counting

10 using a Wallac Microbeta TriLux plate reader.

Each donor was also tested for their ability to respond to two positive control peptides influenza haemagglutinin A amino acids 307-319 [Krieger JJ, et al (1991) *Journal of Immunology*; 146: 2331-2340] and chlamydia HSP60 amino acids 125-140 [Cerrone MC, et al (1991) *Infection and Immunity*; 59: 79-90]. Keyhole limpet haemocyanin, a well documented

15 potent T cell antigen was also used as a control.

Donors that responded to peptides with an SI>2 were analyzed further by plotting the frequency of donor responses to each peptide. Prominent regions of immunogenicity were determined by peptides that induced responses in 10% of donors; however, borderline responses where individual SI values >1.95 were achieved and if two adjacent peptides

20 induced responses in 5% of donors.

In the initial analysis using 25 donor samples, peptides located within two separate regions were able to induce T cell proliferation R1 was located at peptide 10 where donor response

was 8% and R2 located at peptide 15 where the donor response was also 8%. Subsequent analysis using further 30 donor samples failed to maintain the 8% donor response rate for peptide 15 within R2 epitope region (see example 6). The final response rate across a total of 55 donor samples for peptide 15 was 4% (2/55) and R2 was not considered a significant epitope. Figure 1 shows the mature sequence of [Val¹ Val²]-hirudin with regions of immunogenicity highlighted in bold.

EXAMPLE 6

Analysis of immunogenic regions by time-course T-cell assays

Bulk cultures of $2-4 \times 10^6$ PBMC/well were established from 20 healthy donor samples in 24 well plates. Cells were incubated for 6 to 9 days with WT and variant peptides spanning the immunogenic regions (see Table 4). T cell proliferation was assessed by tritiated thymidine incorporation on days 6, 7, 8 and 9. Proliferation was assessed at each time point, by gently resuspending the bulk cultures and removing samples of PBMC, that were then incubated in triplicate wells of U-bottomed 96 well plate with $1 \mu\text{Ci/well}$ tritiated thymidine for 18 hours as described above.

The time course assay was used to test variant peptides containing substitutions over WT. Substitutions were made at key locations where there was expectation that the substitution would prevent binding to MHC class II and therefore, subsequent T cell proliferation in the assay. Figure 2 and Figure 3 respectively show exemplary donor responses over the time course and overall response frequencies. Table 4 shows the sequences of the peptides tested and also the overall response frequencies to each peptide.

The frequency of response is significantly reduced for all substitutions tested. The most effective substitutions are I29A L30A (M 1) and I29R L30H (M 2).

Table 4.

Sequences of peptides used in time-course assays

Wild Type Sequence	% donor response in time course	Modified Sequences	% donor response in time course
CILGSDGEKNQCVTG	25	CAAGSDGEKNQCVTG	5
		CEKGSDEGEKNQCVTG	10
		CRHGSDEGEKNQCVTG	5

EXAMPLE 7**Functional activity of most preferred hirudin muteins**

The thrombin inhibitory activity of the most preferred molecules of the invention was tested. Results are shown in Table 5. Cell-culture supernatants from variants and WT hirudin from
5 transfected HEK.293 cells, were compared in the chromogenic thrombin cleavage assay. The amount of hirudin variants in tissue culture supernatants were quantified by ELISA, and the thrombin inhibition activity was titrated in 2 fold serial dilutions.

The most preferred molecules of the invention demonstrate functional activity within 60% of
10 the WT molecule.

Table 5**Functional activity of most preferred hirudin muteins**

Hirudin variant	Relative activity (ED ₅₀ variant / ED ₅₀ WT hirudin)
I29A L30A (M 1)	1.4
I29R L30H (M 2)	1.2